FUNGI AND YEASTS ISOLATED FROM GREEK TANNERY LIQUID WASTES

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SUMMARY - Greek tannery wastes are relatively rich in organic materials, with mimosa condensed tannins being predominant (6% w/v). In spite of the tannin toxicity for the microorganisms, the microbial flora of tannery liquid wastes is relatively rich, with the *Aspergillus niger* group predominant. Sixteen fungal species belonging to six genera and two yeasts belonging to one genus were isolated. Most of the isolates are adapted to tanninous environments. All isolated species were grown in tannery liquid wastes; 61% of the isolates reduced the tannin content of the waste more than 50%. *Aspergillus carbonarius* presented the highest tannin reduction (78%). The cultivation of the best isolates in tannery liquid wastes gave a biomass rich in protein and a safe culture filtrate, seeing that the initial BOD (3000 mg/l) was reduced to 70 mg/l.

RÉSUMÉ - Les déchets des tanneries grecques sont relativement riches en substances organiques où prédominent les tanins condensés de mimosa (6%). Malgré la toxicité des tanins pour les microorganismes, la flore des microbes des déchets liquides de tanneries est relativement riche avec la prédominance du groupe de *Aspergillus niger*. Seize espèces fongiques appartenant à six genres et deux levures appartenant à un genre ont été trouvées. La plupart des souches sont adaptées aux milieux riches en tanins. Toutes les espèces se sont développées dans les déchets liquides de tanneries. 61% des souches ont réduit le contenu en tanins des déchets de plus de 50%. *Aspergillus carbonarius* a présenté la plus grande réduction des tanins (78%). La culture des meilleures souches dans les déchets liquides des tanneries a donné une biomasse riche en protéines et un filtrat de culture sûr, puisque la DBO initiale (3000 mg/l) a été réduite à 70mg/l.

KEY WORDS - tanneries, tannery wastes, tannins, condensed tannins, mimosa tannins.

INTRODUCTION

Tannins, an important group of natural phenolics, have a toxic effect on the microorganisms and several animals because of their ability to form complexes with other compounds, mainly proteins and polysaccharides (Henis *et al.*, 1964; Grant, 1976). Therefore, enzymes are strongly inactivated either wholly or partially by their binding with tannins, while potential microbial substrates (polysaccharides, nonenzyme proteins) become highly resistant to microbial attack after binding to tannin molecules (Benoit & Starkey, 1968; Benoit *et al.*, 1968). Several of the condensed tannins are themselves very resistant to microbial attack.

Mimosa bark condensed tannins (catechols) are far less liable to deterioration by attack from microorganisms than most natural tanning materials, many of which suffer serious uncontrolled losses from this cause. Thus, mimosa tannins are perhaps the most widely used tanning material in the world. More than 80 countries, including Greece, use mimosa tannins for leather treatment.

The tannery liquid wastes, containing an amount of mimosa tannins, pollute the environment, mainly the aquatic ecosystems.

Despite the detrimental effect of condensed tannins on microbial cell, it is, nevertheless, evident that microorganisms, capable of degrading such an ubiquitous natural product, exist in different environments (Marakis, 1985; Marakis & Karagouni, 1985; Marakis & Diamantoglou, 1990). These organisms, which have been adapted to tanninous environments, contribute significantly to soil biochemistry and generally to the microbial ecology.

The aim of this study was to determine fungi and yeasts occurring on tannery wastes, and to continue our previous efforts (Marakis, 1985; Marakis & Karagouni, 1985; Marakis & Diamantoglou, 1990) to isolate microbial strains with high tanninolytic abilities. Isolations of fungi and yeasts from tannery wastes are undertaken for the first time.

This paper describes: a) isolation and identification of fungi and yeasts occurring in liquid wastes of greek tanneries and b) growth physiology (tanninolytic abilities, etc.) of the isolated microorganisms cultivated in different culture conditions.

MATERIALS AND METHODS

Media

1. For the isolation of microorganisms, many common standard or selective media [Czapek-Dox, potato dextrose, malt extract, corn meal, hay-infusion agar, etc (Miller *et al.*, 1957; Raper & Fennell, 1965; Booth, 1971; von Arx, 1981; Burns & Slater, 1982)] were used. Chloramphenicol (0.05 mg /ml of medium) was added in order to suppress bacterial growth.

2. Medium-a: tannery liquid wastes enforced with (g/l): $(NH_4)_2SO_4$, 5; Na_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; yeast extract, 0.1. *Note*: The addition of yeast extract in the medium did not improve the mycelial growth with an exception of *Penicillium funiculosum*.

3. Medium-b, contained (g/l): mimosa tannins, 20; $(NH_4)_2SO_4$, 5; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $ZnSO_4$, 0.01; $CuSO_4.5H_2O$, 0.005; biotin, 0.04; thiamine, 1; pyridoxine hydrochloride, 0.5 and nicotinic acid, 0.5. The pH was adjusted to 5 - 5.5.

4. Medium-c: its composition was the same with that of the medium-b except that the mimosa tannins were replaced by thioglycolic acid mimosa tannin degradation products. The mimosa tannin degradation was performed by the method of Tamir *et al* (1971).

5. Medium-d, contained (g/l): $(NH_4)_2SO_4$, 5; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 1; $FeSO_4.7H_2O$, 0.05; stearic acid, 30; agar, 15.

The media were sterilized by autoclaving (15 min, 121°C).

Isolation and identification of microorganisms

During the years 1989 - 1992, 500 samples of liquid wastes were obtained from various tanneries of Attica districts and the islands of Samos and Crete. Most of the greek tanneries, which use tanning materials, are installed in these regions. The samplings took place once each season of each year, under aseptic conditions, according to Jones (1971) and Mavridou (1987) procedures.

For the isolation of fungi and yeasts, 1 ml of liquid waste, or of a known dilution of the waste, was mixed with molten agar medium (43°C) into a petri-dish by simultaneous stirring. The streak plate method was used as well. After setting and incubating at ten different temperatures (between 22.5 - 45°C), the growing colonies were purified by several methods (direct isolation, dilution-plate, etc.) as they are described by Raper & Thom (1949), Raper & Fennell (1965), Booth (1971), Waterhouse (1971), Charpentié & Marakis (1980) and Collins *et al.* (1989).

The pure microbial isolates were preserved by lyophilization (Marakis, 1980). These microorganisms were identified according to the classification tables by Raper & Thom (1949), Barnett (1955), Raper & Fennell (1965), Ellis (1971), Ainsworth *et al.* (1973), von Arx (1981), Meyer *et al.* (1987) and Samson & Pitt (1990). No attempt was made to determine relative frequency of occurrence of microbial species in a sample.

Batch cultivation

1. Microbial growth in media-a,b,c: The isolates were grown in 300 ml Erlenmeyer flasks containing 50 ml of medium. These flasks were inoculated with 10^6 fungal spores or yeast cells/ml of medium and incubated on reciprocal shaker (120 strokes per min) for 96h at optimum temperature for each microbial species. Each of the isolates was also cultivated in medium-a at temperature 5°C higher than its optimum temperature of growth. The biomass was harvested by the method of Marakis (1985). Each of the above experiments was run in triplicate (3 flasks per run). The results were presented as mean values ± standard error.

2. Growth on medium-d: The microorganisms were grown in petri-dishes of 9 cm. The colony growth was assessed visually after 96h of incubation.

Analytical methods

- Total nitrogen (TN) was estimated by the method of Varley (1966).
- Protein nitrogen (PN) was calculated by comparing non protein nitrogen (NPN), to TN. For the calculation of NPN, hexosamines and nucleic acids were assumed to contain 7.8 and 15% N, respectively (Smith *et al.*, 1975).
- True protein = PN x 6.25.
- Total lipids were determined by the method of Winter (1963).
- Total tannins were determined according to AOAC (1970) and Marakis (1985).
- BOD_5 and COD were measured by the 507, 508 methods of American Public Health Association (A.P.H.A.) (1975).

RESULTS AND DISCUSSION

Tannin Liquid Wastes (TLW) annual production and their main components

The greek tanneries which use mimosa tannins for sole leather tannages, produce about 2.5×10^7 l/year of liquid wastes.

The composition of TLW is very complex depending on the leather treatment procedure and the good or bad state (quality) of the raw materials (fleeces). The main components of the examined TLW are the following: total mimosa condensed tannins, 2-6%; total lipids, 0.2-0.4%; total nitrogen, 0.3-0.6%; COD, 3000-5500 mg/l; BOD_5, 1500-3000 mg/l of wastes; pH, 6-10. These values of the examined parameters are similar to those reported by Papaconstandinou *et al.* (1993) for the TLW in Samos.

The tannery wastes, relatively rich in organic materials, are discharged in Mediterranean sea causing severe problems to the sea-ecosystem.

Isolated microbial species

The TLW contain condensed tannins toxic and tolerant to microbial attack. Therefore a poor microbial flora was expected in these wastes. On the other hand, during this study, up to 600 isolates were obtained and classified into 18 species belonging to 7 genera (Table I). This is due to the fact that TLW contain -besides tannins- proteins, lipids, etc. which are consumed by tannin-tolerant microorganisms.

The filamentous fungi accounted for the 88.9% on total isolated species. The genus *Aspergillus* appeared at higher frequency (38.9%) than *Penicillium* (22.2%) and yeasts (11.1%).

The species *A. carbonarius, A. phoenicis, A. tenuissima, A. ellipticus, A. flavus, A. japonicus, P. glabrum, P. variotii, A. niger, M. genevensis, C. tropicalis* were isolated from all samples, while *A. alternata, C. guilliermondii and P. oxalicum* were obtained from some samples of TLW. This is somehow enigmatic as *A. alternata* and *P. oxalicum* are cosmopolitan saprophytic colonists of plant surfaces (decaying leaves, fruits, etc.). *A. clavatus* was isolated only from tanneries of Samos. The other isolated species were obtained only from one sample. Therefore it could be supposed that the above first 11 species, which were isolated from all the examined samples, should consist fungal and yeast flora of the tannery wastes. This assumption is supported by the higher biomass dry weight and tanninolytic abilities of these microorganisms compared to those of the other isolated species (Table I).

Table I - Tanin reduction, biomass dry weight and protein contents of the isolated microorganisms cultivated in medium-a, and their growth on medium-d. Incubation time = 96 h.

Tableau I - Réduction des tanins, poids sec de biomasse et contenus en protéines des micro-organismes isolés cultivés dans le milieu-a, et leur croissance sur le milieu-d. Temps d'incubation = 96 h.

Examined parameters / Microorganisms	Biomass dry weight (mg/ml)	Protein content (%)	Percentage of tannin reduction	Growth in medium-d*
1. *Aspergillus carbonarius* Bain.	23.1 ± 0.09	36.6	78.1	+++
2. *Aspergillus phoenicis* (Cda) Tom	20.7 ± 0.14	28.3	70.9	+++
3. *Alternaria tenuissima* (Kunze ex Fr.)Wiltshire	18.8 ± 0.10	26.7	75.4	++
4. *Aspergillus ellipticus* sp. nov.	18.7 ± 0.18	30.1	62.7	++
5. *Aspergillus clavatus* Desm.	18.4 ± 0.10	31.2	31.4	+++
6. *Aspergillus flavus* Link	18.1 ± 0.20	34.1	60.1	++
7. *Aspergillus japonicus* Saito	13.3 ± 0.06	25.2	61.3	++
8. *Penicillium glabrum* (Weh) West.	13.1 ± 0.03	32.8	73.2	++
9. *Paecilomyces variotii* Bain.	11.9 ± 0.06	35.2	51.1	++
10. *Aspergillus niger* Van Tiegh.	10.7 ± 0.05	31.1	50.3	+++
11. *Mucor genevensis* Lendner	8.5 ± 0.15	33.4	37.1	++++
12. *Phomopsis oblonga* Desm.	8.1 ± 0.23	30.9	60.5	+
13. *Penicillium funiculosum* Thom	7.8 ± 0.21	37.2	2.8	++++
14. *Penicillium expansum* Link	7.7 ± 0.10	30.7	28.7	++
15. *Penicillium oxalicum* Cur.& Thom	6.5 ± 0.08	34.2	37.4	+
16. *Alternaria alternata* (Fr.) Keis.	6.3 ± 0.10	32.0	32.2	++
17. *Candida tropicalis* (Cast) Berkh.	4.2 ± 0.05	40.1	59.5	+
18. *Candida guilliermondii* (Cast) Lang	2.8 ± 0.07	44.9	30.3	-

* Growth assessed visually: -, none; +, poor; ++, moderate; +++, good; ++++, luxuriant.

Although *P. funiculosum* presented a relatively good mycelial growth, it did not consume tannins in medium-a. This fungal species is possibly an airborne tannin-tolerant invader, which was fortuitously found in only one tannery waste sample.

Biomass and protein content

As Table I shows, biomass dry weight ranges between 2.8-23.1 mg/ml of medium. Filamentous fungi, especially *A. carbonarius* and *A. phoenicis*, showed higher biomass production compared to yeasts. On the other hand the yeast biomass was richer in protein (40-45% on dry weight) than that of filamentous fungi (25-36%). This should be expected because yeasts are considered to be among the protein-rich micro-organisms.

The mycelial protein contents of the examined species are considered to be high in reference to filamentous fungi in general. Thereafter, microorganisms of the best biomass and protein production can be cultured in TLW in order to produce a biomass rich in protein, which, after proper chemical analyses and nutritional tests, should be used for animal feed. This way, most of the tannery liquid waste organic materials will be converted into microbial biomass resulting in an almost clear liquid residue with a very low BOD. The mixed culture of *A. carbonarius* and *P. glabrum* reduced the tannery wastewater BOD_5 from 3000 to 70 mg/l after 96h of cultivation.

Tannin reduction

A. carbonarius presented the highest tannin reduction (78% on initial tannins) while *P. funiculosum* presented the smallest one (2.8%) (Table I). Marakis (1980; 1985) reported that *A. carbonarius* strains (AsDT10, Asca1) presented a high tanninolytic ability in carob tannin substrates. A high tannin reduction percentage (75%) was also observed in *A. tenuissima* culture. Motoda (1978) determined polyphenol oxidase in synthetic medium culture of *A. tenuis* strain (A-2). *C. tropicalis* presented a relatively high tannin reduction. Ötük and Deschamps (1983) reported that *C. guilliermondii* and *C. tropicalis* strains presented a rapid degradation of mimosa condensed tannins. Our previous studies on microbial screenings for tannin degradation, fungal protein production, etc. (Charpentié & Marakis, 1980; Marakis, 1985; Marakis & Diamantoglou, 1990) showed that strains of *A. carbonarius* possess the strongest condensed tannin degradation system of all the microorganisms examined so far. This fungal species has been isolated, till now, from all the examined tanninous environments in which it is well adapted. A research on condensed tannin degradation enzyme system of *A. carbonarius* is carried out in our laboratory, nowadays, by Solid State Fermentation technique.

The 1/3 of the isolates presented a tannin reduction which is in excess of 60% of the substrate tannins. The remaining (2/3) isolated tannin-tolerant species which use smaller tannin amounts, appear to prefer other carbon sources (proteins, lipids, etc.) contained in TLW.

The biomass dry weight differences between the examined microbial species do not correspond to those of tannin reduction percentages. For example, although the biomass of *A. tenuissima* was similar to that of *A. clavatus*, the tannin reduction

percentage in the culture of the former fungus was 140% higher than that of the latter one. Nevertheless, the reverse figure was also observed. So, although the fungal pairs *A. flavus* - *P. oblonga* and *A. tenuissima* - *P. glabrum* presented similar tannin reduction, the biomass dry weights differed (44-123 %). This differentiation of the biomass dry weight and tannin reduction should be due to the different quantitative utilisation of tannery waste ingredients (tannin, proteins, lipids, etc.) by the isolated microorganisms. Therefore *P. funiculosum* growth, which is supported by lipids, proteins, etc. is not affected by the tannin presence. A strain of this fungal species, which had been isolated from leaves of *Olea europaea* var. *silvestris*, failed to grow on substrates which contained condensed tannins as sole carbon source (Marakis & Diamantoglou, 1990).

Fungal species, which reduced the tannin content more than 50%, in medium-a, were grown in medium-b, containing mimosa tannins as sole carbon source. Most of these species have been also isolated from tanninous materials (fruits, leaves, etc.) (Charpentié & Marakis, 1980; Marakis & Diamantoglou, 1990). Consequently these fungi, especially *A. carbonarius, A. phoenicis,* and *A. tenuissima*, should be adapted to tanninous environments. *A. phoenicis* and *A. tenuissima* were isolated for the first time, from tanninous materials. These species have to be included into the tannin-utilizing group of microorganisms because of their ability to grow in medium-b.

The species *A. clavatus, A. alternata, P. oxalicum* and *P. funiculosum* were not grown in medium-b. However, they presented (except *P. funiculosum*) a relatively rich mycelial growth in medium-c, containing mimosa tannin thioglycolic acid degradation products. These species were grown in medium-a and reduced the tannin content more than 30% (Table I). This means that these fungi require, at least, a more readily utilizable carbon source (proteins, lipids, etc.) for the condensed tannin degradation. The treatment of the tannery liquid wastes with thioglycolic acid should also benefit the fungal growth by converting the toxic tannins into biomass which can be removed from the waste by filtration, minimizing, this way, the TLW organic materials.

The tannin reduction percentage was almost doubled by *P. variotii, P. oblonga, M. genevensis* and *A. alternata* in medium-a at an incubation temperature of 5°C higher than their optimum culture temperature which was determined in Czapek-Dox-agar medium. This is possibly due to the fact that the tannin degradation enzyme system becomes more active under the higher incubation temperature than the optimum one for the mycelial growth in the synthetic medium. However, this was not observed in other isolated species.

Growth on medium-d

The isolated microorganisms, except *C. guilliermondii*, presented a growth differentiation on medium-d (Table I). *P. funiculosum* and *M. genevensis* presented the most abundant growth, followed by *A. carbonarius, A. phoenicis, A. niger* and *A. clavatus*. These fungal species, mainly the two former ones, should possess higher lipolytic abilities than the ones of the other isolated species. This can be supported by the fact that *P. funiculosum* presented a relatively good growth in medium-a, consuming tannery waste lipids and/or other substances except tannins.

CONCLUSIONS

- The fungal flora of TLW can be considered relatively rich.
- Most of the isolated species appear to be adapted to such tanninous environments.
- Depending on the culture conditions, a differentiation of physiological activities of the isolated microorganisms was observed.
- Isolated species, which utilize not only tannins but protein and lipids as well, can be considered as microorganisms of ▪ great ecological importance, because:

a) They degrade the tolerant against microbial attack, mimosa tannins.

b) The conversion of the tannery waste organic materials into fungal biomass will result into a safe wastewater. It is noticeable that the installation of all tanneries of each district at one place and the valorization of the fungal biomass for animal feed will possibly become profitable for the detoxification of the tannery liquid wastes through this procedure.

REFERENCES

AINSWORTH G.C., SPARROW F.K. and SUSSMAN A.S., 1973 - A taxonomic review with keys: Ascomycetes and Fungi Imperfecti. *In*: AINSWORTH G.C., SPARROW F.K. & SUSSMAN A.S., *The fungi* Vol. IV A. New York and London, Academic Press.

A.O.A.C., 1970 - *Official methods of Analysis of the Association of Official Agricultural Chemists.* 11th ed., Washington, Horwitz W. Ed.

A.P.H.A., 1975 - *American Public Health Association Standard Methods for the examination of Water and Wastewater.* 14th Ed. pp. 543-554, New York.

ARX J.A. von, 1981 - *The genera of fungi sporulating in pure culture.* Vaduz, J. Cramer.

BARNETT H.L., 1955 - *Illustrated genera of imperfect fungi.* Minneapolis, Burgess Publishing Co.

BENOIT R.E. and STARKEY R.L., 1968 - Enzyme inactivation as a factor in the inhibition of decomposition of organic matter by tannins. *Soil Sci.* 105 (4): 203-208.

BENOIT R.E., STARKEY R.L. and BASARABA J., 1968 - Effect of purified plant tannin on decomposition of some organic compounds and plant materials. *Soil Sci.* 105 (3): 153-158.

BOOTH C., 1971 - Introduction to general methods, fungal culture media. *In*: BOOTH C., *The methods in Microbiology.* London & New York , Academic Press, Vol. 4: 1-94.

BURNS R.G. and SLATER J.H., 1982 - Procedures for the isolation, cultivation and identification of fungi.: *In* BURNS R.G. & SLATER J.H., *Experimental Microbial Ecology.* London, Blackwell Sci. Publ.: 22-30.

CHARPENTIÉ M.J. et MARAKIS S., 1980 - La mycoflore des caroubes. *Cryptog. Mycol.* 1: 165-174.

COLLINS C.H., LINE P.M. and GRANGE J.M., 1989 - Cultural, Identification, Mycological, methods. *In*: COLLINS C.H. and LINE P.M., *Microbiological methods.* London, Boston, Singapore, Toronto, Butterworths: 84-126.

ELLIS M.B., 1971 - The genus *Alternaria. In*: ELLIS M.B. *Dematiaceous hyphomycetes.* Cab International, Wallingford U.K.: 464-497.

GRANT W.D., 1976 - Microbial degradation of condensed tannins. *Science* 193: 1137-1138.

HENIS Y., TAGARI H. and VOLKANI R., 1964 - Effect of water extracts of carob pods, tannic acid and their derivatives on the morphology and growth of microorganisms. *Appl. Microbiol.* 12 (3): 204-209.

JONES E.B.G., 1971 - Aquatic fungi. *In*: BOOTH C., *The methods in Microbiology*. London, New York, Academic Press, Vol. 4: 335-365.

MARAKIS S., 1980 - "New fungal strains for microbial protein production from carob beans". Ph. D. Thesis, Univ. of Athens (in greek).

MARAKIS S, 1985 - Screening tannin-utilizing filamentous fungi for protein production from aqueous carob extract. *Cryptog. Mycol.* 6: 293-308.

MARAKIS S. and KARAGOUNI A., 1985 - Screening of carob bean yeasts. Chemical composition of *Schizosaccharomyces versatilis* grown on aqueous carob extract. *Biotechnol. Lett.* 7: 831-836.

MARAKIS S. and DIAMANTOGLOU S., 1990 - Fungi isolation from leaves of some mediterranean evergreen sclerophyllous shrubs. Enzymatic activity of the isolated fungi. *Cryptog. Mycol.* 11 (4): 243-254.

MAVRIDOU A., 1987 - Microbiological study of sea water in relation to: pollution, chlorophylls, temperature and other factors. Ph.D. Thesis. Univ. Athens (in greek).

MEYER S.A., AHEARN D.G. and YARROW D., 1987 - Genus 4. *Candida* Berkhout. *In:* KREGER-VAN RIJ N.J.W., *The yeasts a taxonomy study*. Elsevier Science Publishers B.V. - Amsterdam: 585-844.

MILLER J.H., GIDDENS J.E. and FOSTER A.A., 1957 - A survey of the fungi of forest and cultivated soils of Georgia. *Mycologia* 49: 779-808.

MOTODA S., 1978 - Effect of yeast extract on production of polyphenol oxidase by *Alternaria tenuis*. *J. Ferment. Technol.* 56 (5): 543-545.

ÖTÜK G. and DESCHAMPS A.M., 1983 - Dégradation d'un tanin condensé par plusieurs types de levures. *Mycopathologia* 83: 107-111.

PAPACONSTANDINOU D., VALERI D. and REPAS D., 1993 - Wastewater treatment for the tanneries on the island of Samos. *In:* ASSOCIATION HELLENIQUE DES TECHNICIENS ET CHIMISTES DE L'INDUSTRIE DU CUIR, 5° Congrès Méditerranéen des techniciens et chimistes des industries du cuir (Grèce, Athènes, 17-20 Mars) : 28-30.

RAPER K.B. and THOM C., 1949 - *A manual of the Penicillia*. London, Balliere, Tindall & Cox.

RAPER K.B. and FENNELL D.I., 1965 - *The genus Aspergillus*. Baltimore, Williams & Wilkins Co.

SAMSON R.A. and PITT J.I., 1990 - *In*: SAMSON R.A. and PITT J.I., *Modern concepts in Penicillium and Aspergillus classification*. Plenum Press New York and London Published in cooperation with NATO Scientific Affairs Division.

SMITH R.H., PALMER R. and READE A.E., 1975 - A chemical and biological assessment of *Aspergillus oryzae* and other filamentous fungi ■ protein sources for simple stomached animals. *J. Sci. Food Agric.* 26: 785-795.

TAMIR M., NACHTOMI E. and ALUMOT E., 1971 - Degradation of tannins from carob pods (*Ceratonia siliqua*) by thioglycolic acid. *Phytochemistry* 10: 2769-2774.

VARLEY J.A., 1966 - Automatic methods for the determination of nitrogen, phosphorus and potassium in plant material. *The Analyst* 91: 119-126.

WATERHOUSE G.M., 1971 - Phycomycetes. *In*: BOOTH C., *The methods in Microbiology*. London & New York, Academic Press, Vol. 4: 183-192.

WINTER E.Z., 1963 - Über ein neues Verfahren zur Bestimmung und Untersuchung von Fetten in Lebensmitteln. *Lebensmittel-Unters. & Forsch.* 123: 205-210.